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The Role of the Prothoracic Gland in Determining Critical Weight for Metamorphosis in *Drosophila melanogaster*

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Summary

Background: The timely onset of metamorphosis in holometabolous insects depends on their reaching the appropriate size known as critical weight. Once critical weight is reached, juvenile hormone (JH) titers decline, resulting in the release of prothoracicotropic hormone (PTTH) at the next photoperiod gate and thereby inducing metamorphosis. How individuals determine when they have reached critical weight is unknown. We present evidence that in *Drosophila*, a component of the ring gland, the prothoracic gland (PG), assesses growth to determine when critical weight has been achieved.
Results: We used the GAL4/UAS system to suppress or enhance growth by overexpressing PTEN or Dp110, respectively, in various components of the ring gland. Suppression of the growth of the PG and CA, but not of the CA alone, produced larger-than-normal larvae and adults. Suppression of only PG growth resulted in nonviable larvae, but larvae with enlarged PGs produced significantly smaller larvae and adults. Rearing larvae with enlarged PGs under constant light enhanced these effects, suggesting a role for photoperiod-gated PTTH secretion. These larvae are smaller, in part as a result of their repressed growth rates, a phenotype that could be rescued through nutritional supplementation (yeast paste). Most importantly, larvae with enlarged PGs overestimated size so that they initiated metamorphosis before surpassing the minimal viable weight necessary to survive pupation.
Conclusions: The PG acts as a size-assessing tissue by using insulin-dependent PG cell growth to determine when critical weight has been reached.

Introduction

Adult size in insects is determined by the final size achieved during the larval growth phase and is fixed once the larva enters metamorphosis. To regulate this transition between growth and metamorphosis, insects use a number of important size-assessment milestones [1]. The first, termed the threshold size for metamorphosis, involves assessing size to determine whether the next molt will be a larval or a metamorphic molt [2]. In *Drosophila*, it occurs late in the second instar (L2) after the molt to the third instar (L3) is initiated [3]. The next size milestone occurs early in the last larval stage and corresponds to the minimal viable weight necessary for a larva to survive to metamorphosis when starved [2]. The final transition between the growth phase and the initiation of metamorphosis requires the attainment of a species-specific critical weight during the final larval stage [1, 4]. We have investigated how body size is assessed in *Drosophila melanogaster* during this last transition and how this mechanism relates to size assessment at the other two size milestones.

In *Drosophila*, larvae starved before attainment of critical weight, then refed, delay metamorphosis for longer than the length of the starvation period [5, 6]. Starvation after critical weight is surpassed does not delay metamorphosis. Thus, larval growth can be characterized by two phases: a pre-critical-weight phase that can vary in length, and a post-critical-weight phase where starvation can no longer delay metamorphosis [6].

Surpassing critical weight results in the initiation of an endocrine cascade for metamorphosis, as originally described for the tobacco hornworm, *Manduca sexta* [7, 8]. Once *Manduca* larvae reach critical weight, juvenile hormone (JH) titers decline, allowing release of prothoracicotropic hormone (PTTH), which acts on the prothoracic gland (PG) to cause synthesis of the molting hormone ecdysone. Ecdysone and its active metabolite 20-hydroxyecdysone (20E) (referred to collectively as ecdysteroids), in the absence of JH, cause commitment to metamorphosis and cessation of feeding. The amount of time required for JH clearance, PTTH induction, and ecdysone synthesis determines the length of the growth period between the achievement of critical weight and initiation of metamorphosis and thus, along with growth rate [9], is vital to determining adult size.

Recent studies in *Drosophila* have revealed that the *Drosophila* insulin receptor (InR), known for its role in nutrition-dependent growth [10], plays different roles in pre-critical-weight and post-critical-weight larval growth [11]. Pre-critical-weight InR activity affects development time but not body size, whereas in the post-critical-weight period, it affects body size but not development time. Thus, the InR cascade appears to be involved in regulating the developmental delays described in starved pre-critical-weight larvae [5, 6] and is downstream of the size-assessment event occurring at critical weight.

The *Drosophila* insulin-like peptides (dilps) are the likely ligands for InR [12, 13]. InR in turn activates its substrate, CHICO, which recruits Dp110 (PI3 kinase) to the membrane via its adaptor protein p60 [14, 15]. Dp110 converts phosphatidylinositol-4,5-bisphosphate (PIP_2) to PI-3,4,5-triphosphate (PIP_3) [15]. The accumulation of PIP_3 in the membrane results in increased cell growth [16]. PTEN is a phosphatase that converts PIP_3 to PIP_2, thus suppressing cell growth [16–20]. The balance between cellular levels of PIP_3 and PIP_2, regulated by Dp110 and PTEN, makes tissues larger or smaller in a cell-autonomous manner.

How nutrition-dependent growth, modulated by the InR cascade, interacts with the size-assessment mechanism that determines critical weight is unclear. However, insulin-like peptides regulate ecdysone secretion in the ovaries of the mosquito *Aedes aegypti* [21] and

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the blow fly *Phormia regina* [22]. In cultured wing discs of the butterfly *Precis coenia*, both bombyxin, the lepidopteran insulin-like peptide, and 20E act synergistically to induce cell proliferation [23]. This crosstalk between the growth and metamorphic pathways could be important for the size-assessment mechanism.

The only insect clade for which the size-assessment mechanism has been identified is the Hemiptera, or true bugs. In the milkweed bug, *Oncopeltus fasciatus*, size is assessed via an abdominal stretch receptor that induces metamorphosis once sufficient mass has been gained [24]. Although this simple, elegant mechanism of size assessment is not found outside the Hemiptera, one could postulate that in other insects, size is monitored by some other size-assessing tissue, possibly via the intrinsic detection of increasing levels of growth factors or growth-factor-activated cascades (also see [11]). If such a size-assessing tissue exists, then experimentally increasing its growth would result in early commitment to metamorphosis and would produce an animal of subnormal size. Conversely, suppressing its growth would result in an extended larval growth period and metamorphosis at larger-than-normal size. We show here that the PG acts as a size-assessing tissue through intrinsic assessment of insulin-pathway-induced growth.

Results

We tested the hypothesis that a tissue within the endocrine or neuroendocrine system may act as a size-assessing tissue by using the GAL4/UAS system of targeted gene expression [25] to enhance (UAS Dp110) or suppress (UAS PTEN) the growth of tissues of interest. Our initial screen identified the ring gland, a composite organ that includes the corpora allata (CA), the prothoracic gland (PG), and the corpora cardiaca, as a likely candidate.

Three GAL4 lines were used to target gene expression in ring-gland tissues (Figure 1). P0206 GAL4 (P0206) is expressed moderately throughout the PG and strongly in the CA (Figures 1A–1C). Aug21 is expressed in the CA and in salivary glands (Figures 1D–1F). Phantom is a P450 enzyme involved in ecdysone biosynthesis [26], and the phantom GAL4 line (phm) is expressed strongly in the PG (Figures 1G-1I). Phm is also transiently expressed in the notum of the wing imaginal disc and in a patch in the presumptive thorax of the leg disc in wandering L3 larvae (data not shown).

Two control genotypes were examined for P0206 and phm. The controls phm>GFP and P0206>GFP were generated by crossing females from the parent stock (either *yw;P0206,UAS mCD8::GFP* or *yw;phm,UAS mCD8::GFP/TM6B Tb*) to *yw* males. Controls carrying two copies of GFP were either the parent stock itself, in the case of P0206x2>GFPx2, or were generated by crossing P0206 or phm females to *yw;UAS mCD8::GFP* males (P0206>GFPx2 and phm>GFPx2). There were significant differences in adult size between the two control groups. Phm>GFP males had smaller wing areas than phm>GFPx2 (Table 1), and for P0206>GFP, both sexes had smaller wing areas compared to P0206x2>GFPx2 (Table 1). These differences in size between the control groups are likely caused by genetic background. Lastly, the UAS PTEN and UAS Dp110 lines showed no noticeable differences in size.

Effects of Suppressing PG Growth on Body Size

The use of P0206 to drive UAS PTEN suppressed the growth of both the PG and CA (Figures 2A and 2B). Sixty-six percent of the P0206>PTEN larvae underwent precocious metamorphosis and formed L2 puparia (n = 76, Figure 2C). Pupariation is unique to higher Diptera and occurs when the postfeeding larva ceases wandering, everts its spiracles, contracts, and tans its larval cuticle to form a protective puparial case [27]. The pupa forms 12 hr later at head eversion. None of the L2 puparia eclosed as adults, although a few (<1%) developed to the pharate adult stage (Figure 2C). The remainder of the P0206>PTEN larvae formed L3 puparia, which as pharate adults were 42% heavier than the controls (Figure 2C). The adult wing areas were 126% (males) and 115% (females) the size of controls (Figure 2D, Table 1).

To determine when this size difference was first manifest, we measured the length of the larval mouth hooks from the intersection at the lateral arch to the tip. Mouth-hook length reflects the amount of growth that occurred in the previous stage—i.e., the first instar (L1) length reflects embryonic size, and the L2 and the L3 lengths are measures of growth in the L1 and L2, respectively. L1 mouth-hook length was indistinguishable between genotypes (Table 2), but P0206>PTEN larvae had significantly larger L2 mouth hooks than the controls (Table 2).

P0206>PTEN larvae were also developmentally delayed, molting to L2 later than control larvae. By 48 hr after egg laying, 35% of P0206>PTEN larvae (n = 23) had molted to L2 as compared to 90% (n = 30) for controls. The P0206>PTEN L2 larvae either molted to the L3 stage 2.7 days after L2 ecdysis or formed precocious L2 puparia 3 days after L2 ecdysis. Control larvae spent 1 day as L2-stage larvae.

Whether a P0206>PTEN larvae forms a L2 puparium at the second molt may depend on whether it crosses the threshold weight for metamorphosis before the ecdysteroid titer rises to induce the L3 molt [3]. To test this hypothesis, we reared P0206>PTEN larvae on food of different qualities, reasoning that slower-growing, undernourished larvae would form L3 puparia at higher frequencies. When P0206>PTEN larvae were reared on cornmeal/molasses medium supplemented with yeast paste, 66% (n = 76) formed precocious L2 puparia. When reared on the same food without yeast supplement, fewer larvae became precocious L2 puparia (57%, n = 49). On food containing 50% of the normal nutrients, none became L2 puparia, and all larvae formed large L3 puparia (n = 77).

Suppressing the growth of the PG alone in phm>PTEN larvae resulted in animals that died after a prolonged L1 (3–4 days). Reducing GAL4 activity by rearing phm>PTEN larvae at 18°C produced the same result. To determine whether the inability of phm>PTEN larvae to molt was due to insufficient ecdysteroid production, we fed them food containing 1 μg/ml 20E. At 25°C, only a few of the larvae fed 20E were able to molt to L2. If,

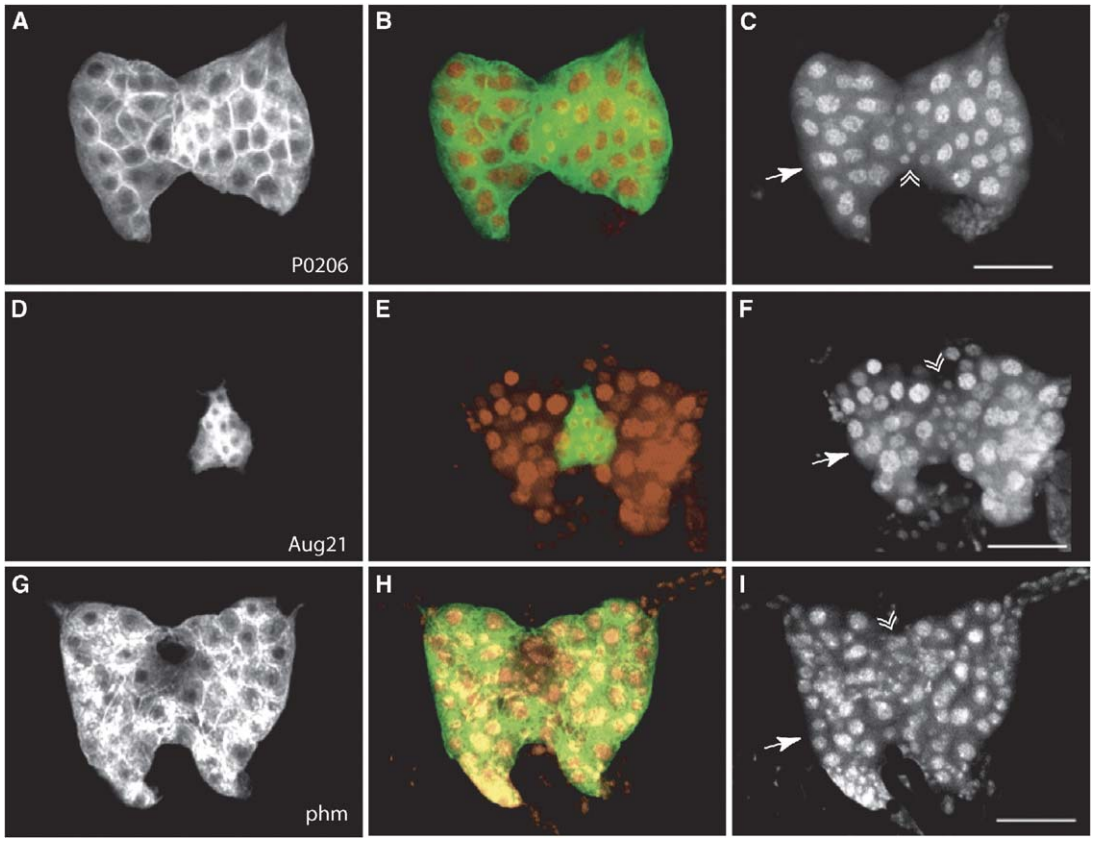


Figure 1. P0206, Aug21, and Phantom (phm) Are Expressed in Different Subsets of Ring-Gland Tissues

The lefthand column (A, D, and G) shows the GFP expression driven by the three GAL4 lines. The righthand column (C, F, and I) shows the same ring glands stained with propidium iodide to mark nuclei, and the middle column (B, E, and H) is the merge between GFP and propidium iodide.

(A–C) P0206 drove expression in both the prothoracic gland (PG, the large nuclei in [C] [arrow]) and the corpora allata (CA, the smaller nuclei in [C] [double arrowhead]).

(D–F) Aug21 was expressed in the CA (double arrowhead in [F]), but not in the PG (arrow in [F]).

(G–I) Phantom (phm) drove expression in the PG cells (arrow in [I]), but not in the CA (double arrowhead in [I]). Scale bars = 50 μm.

however, they were kept at 18°C and fed 20E for 22 hr, beginning 4 days after oviposition, approximately 34% (n = 90) successfully molted to L2. When these L2 larvae were refed on 20E-containing food 4 days after the molt to L2, 27% made precocious L2 puparia, and 7% molted and metamorphosed as L3 prepupae. The difference in effects between phm>PTEN and P0206>PTEN larvae was likely caused by differences in driver strength; phm is a stronger driver in the PG than P0206 (compare GFP intensity in Figures 1A and 1G).

Reducing the size of the CA alone (Aug21>PTEN) had no effect on pharate adult weight or on adult wing area (Table 1).

Effects of Enhancing the Growth of the PG on Body Size

When Dp110 was expressed specifically in the PG by using phm, the PG cells were enlarged during all feeding stages (L1, Figures 3A and 3B; early feeding L3, Figures 3C and 3D). The resulting phm>Dp110 pharate adults were 73% and 80% (males and females, respectively) the weight of phm>GFP controls (Figure 3E; Table 1). Eclosed phm>Dp110 adults also had smaller wing areas than controls (Figure 3E, Table 1).

At hatching, both phm>Dp110 and phm>GFP larvae had mouth hooks of the same size (Table 2). However, phm>Dp110 larvae grew less in the L1 and L2, as indicated by the smaller size of the L2 and L3 mouth hooks (Table 2). No difference in duration of either the L1 or L2 was detected between phm>Dp110 larvae and controls. However, phm>Dp110 larvae reared without yeast supplement spent less time in L3, pupariating 46 ± 0.8 hr after L3 ecdysis (AL3E). Phm>GFP and phm>GFPx2 controls pupariated 59 ± 0.9 and 72 ± 0.9 hr AL3E, respectively.

There were no significant differences seen between P0206>Dp110 and control pharate-adult weights or adult wing areas (Table 1). Presumably, P0206 is not a strong enough driver to produce effects on body size under these conditions. Also, increasing the size of the CA alone, by using Aug21, had no effect on either pharate-adult weight or adult wing area (Table 1).

The Role of Photoperiod in Size Assessment

In Lepidoptera, once critical weight is reached, JH titers decline, allowing PTTH to be secreted [3]. Post-critical-weight secretion of PTTH in *Manduca* is under circadian control; thus, it only occurs during the next circa-

Table 1. The Effects of Changing Ring-Gland Size, by using the P0206, Phantom, and Aug21 GAL4 Drivers, on Pupal Weight and Wing Area in Males and Females

	Male			Female		
	N	Pharate-Adult Weight (mg)	Wing Area (mm^2)	N	Pharate-Adult Weight (mg)	Wing Area (mm^2)
P0206: PG and CA						
P0206>GFP	27	1.18 ± 0.01^A	1.30 ± 0.01^A	34	1.53 ± 0.01^A	1.74 ± 0.01^A
P0206x2>GFPx2	22	1.26 ± 0.02^A	1.47 ± 0.01^B	22	1.58 ± 0.02^A	1.85 ± 0.01^B
P0206>Dp110	23	1.19 ± 0.02^A	1.36 ± 0.02^C	23	1.52 ± 0.02^A	1.83 ± 0.01^B
P0206>PTEN	20	1.82 ± 0.08^B	1.64 ± 0.02^D	13	2.04 ± 0.1^B	2.00 ± 0.03^C
phm: PG						
phm>GFP	33	1.36 ± 0.02^A	1.36 ± 0.01^A	33	1.76 ± 0.02^A	1.79 ± 0.01^A
phm>GFPx2	20	1.39 ± 0.03^A	1.46 ± 0.03^B	37	1.75 ± 0.03^A	1.84 ± 0.01^A
phm>Dp110	67	0.99 ± 0.02^B	1.19 ± 0.01^C	46	1.41 ± 0.02^B	1.70 ± 0.02^B
Aug21: CA						
Aug21>GFP	28	1.13 ± 0.01^A	1.41 ± 0.01^A	24	1.38 ± 0.02^A	1.81 ± 0.01^A
Aug21>Dp110	32	1.27 ± 0.03^A	1.39 ± 0.01^A	26	1.38 ± 0.03^A	1.82 ± 0.01^A
Aug21>PTEN	17	1.05 ± 0.03^A	1.39 ± 0.03^A	16	1.34 ± 0.02^A	1.78 ± 0.02^A

N is the number of individuals scored. The first row for each GAL4 line indicates in which tissues GAL4 drives its expression. PG is the prothoracic gland, and CA is the corpora allata. Means not connected by the same letter (uppercase superscript) were found to be statistically significant at $p < 0.05$ by ANOVA and a post-hoc comparison of the means, with a Tukey HSD. The values after the means are the standard errors of the means.

dian gate after the JH titers have dropped [4]. In the interval between critical weight and PTTH secretion, feeding and growth continue; this interval is called the PTTH delay period [7]. Once PTTH is secreted, the resulting pulse of ecdysteroids induces wandering behavior, marking the end of growth.

Although PTTH secretion has not been directly observed in *Drosophila*, the onset of wandering behavior is under circadian control [28]. Circadian gating can be abrogated by rearing animals under constant light (LL) conditions, which render *Drosophila* arrhythmic. Therefore, we reared larvae either under 14 hr light, 10 hr dark (14L:10D) conditions or under LL to determine whether size would be affected by photoperiod. The pharate-adult weight of the P0206 and phm controls was unaffected by photoperiod, except for P0206>GFP, which was significantly smaller under LL (Figures 4A and 4B).

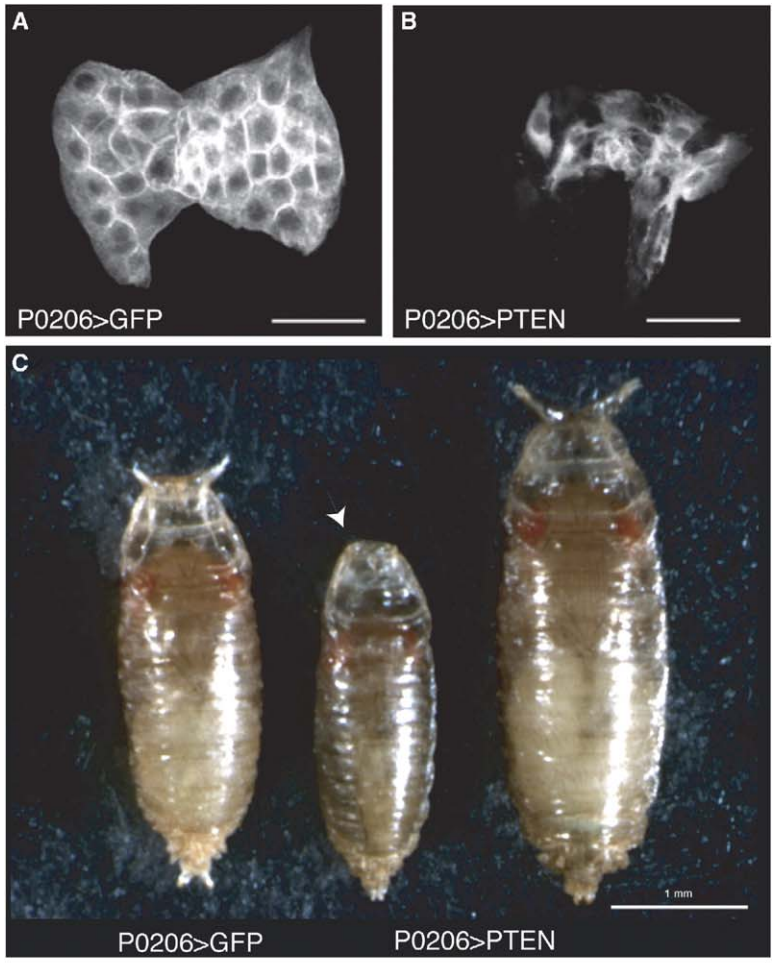
In contrast to the controls, larvae with enlarged prothoracic glands formed smaller pupae when reared under LL (Figures 4A and 4B). P0206>Dp110 pharate adults reared under LL were 80% the size of P0206>GFP pharate adults grown under the same conditions (n = 40 each) (Figure 4A). Phm>Dp110 pharate adults raised under LL were even smaller (51% of phm>GFP controls) (Figure 4B) and pupariated 4 ± 1.0 hr earlier than phm>Dp110 larvae reared under 14L:10D. When phm was used to drive InR (phm>InR), the resulting pharate adults weighed 42% of the weight of phm>GFP controls under LL conditions.

Minimal Viable Weight and Critical Weight for Metamorphosis in phm>Dp110 and phm>GFP Larvae

Our hypothesis is that phm>Dp110 animals, as a result of their enlarged PGs, attain critical weight for metamorphosis at smaller sizes. Alternatively, phm>Dp110 adults may be smaller because enlarging the PG affects growth in a manner unrelated to the attainment of critical weight. To distinguish between these hypotheses, we determined growth rates and critical weight for control and phm>Dp110 larvae.

Recent studies of the role of Lk6 kinase in insulin-mediated size control show that nutritional conditions can greatly affect the outcome of results relating to size [29, 30]. Therefore, we constructed growth curves for phm>Dp110 and controls reared under LL with two different nutritional conditions: (1) standard cornmeal/molasses medium supplemented with yeast paste, and (2) standard medium alone. When reared under LL on a yeast-supplemented diet, the phm>Dp110 larvae were 82% the weight of control larvae at the time of ecdysis to L3. Nevertheless, under these conditions, phm>Dp110 larvae grew at the same rate as control larvae (Figure 5A). Without yeast supplement, phm>Dp110 larvae ecdysed at even smaller sizes (65% of the controls, Figure 5B) and grew at a slower rate than controls (Figure 5B).

The critical weight for metamorphosis was determined by starving L3 larvae of known weight and time classes and then determining the time to pupariation. Figure 6A shows that 50% threshold for pupariation for starved phm>GFP control larvae was reached at 11.4 hr AL3E at a weight of 0.86 mg. Phm>GFPx2 larvae attained this threshold at 9.2 hr AL3E at a weight of 0.92 mg. In contrast, 50% of the phm>Dp110 larvae were able to pupariate after starvation at 2.6 hr AL3E when they were only 0.36 mg. At this early time, larvae that did not form normal puparia showed partial pupariation, characterized by tanning of the larval cuticle and extension of posterior spiracles, but failed to evert their anterior spiracles and form an operculum (Figure 6C, 0 hr AL3E). Six of the 15 phm>Dp110 larvae starved at the time of ecdysis formed such partial puparia; the remainder died as larvae. Of the larvae starved after 4 hr AL3E, 73% (n = 15) formed normal puparia, 13% formed partial puparia, and the remaining larvae died. When starved at later times, phm>Dp110 larvae only formed



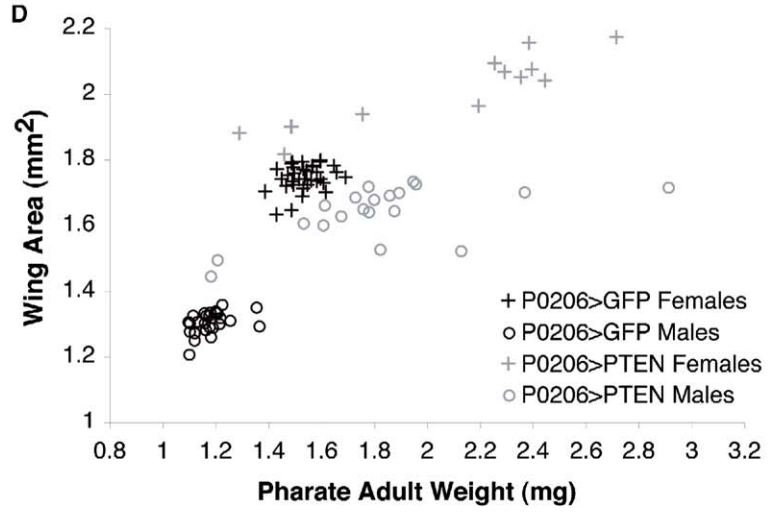


Figure 2. Suppressing the Growth of the Ring Gland Results in L2 Pharate Adults and Large L3 Pharate Adults

(A and B) GFP expression in the ring glands of both control third instar (L3) larvae (P0206>GFP, [A]) and P0206>PTEN L3 larvae (B).

(C) Pharate adults formed from control P0206>GFP individuals (left) and P0206>PTEN larvae (L2 and L3 pharate adults, middle and right, respectively). The L2 pharate adult was identified by the club-shaped anterior spiracle morphology characteristic of L2 larvae (arrow, [27]).

(D) Adult wing area as a function of pharate adult weight for P0206>GFP control and P0206>PTEN (L3 pharate adults) males and females. The scale bars represent 50 μm in (A) and (B) and 1 mm in (C).

normal puparia. The phm>GFP and phm>GFPx2 control larvae never formed partial puparia.

Minimal viable weight is the minimal weight required for 50% of the larvae to survive to pupation when starved [2]. Most phm>GFP and phm>GFPx2 larvae that were able to pupariate after starvation in the above experiment also underwent head eversion and adult development (50% threshold = 0.88 mg at 11.6 hr AL3E and 0.92 mg at 9.2 hr AL3E, respectively). In contrast, phm>Dp110 larvae that pupariated when starved at very early times were nonviable. These larvae only attained the 50% pupation rate around 11.5 hr AL3E, similar to the time seen for control larvae (Figure 6B). Importantly, the size of phm>Dp110 larvae at this time was 0.52 mg, which is smaller than the controls.

Why phm>Dp110 larvae were unable to pupate when

Table 2. The Effects of Changing Ring-Gland Size, by using the GAL4 Lines P0206 and phm, on Mouth-Hook Length in First (L1), Second (L2), and Third (L3) Instar Larvae

	Mouth-Hook Length (mm)		
	L1	L2	L3
P0206: PG and CA			
P0206>GFP	0.108 ± 0.001 (30)^{A}	0.167 ± 0.001 (29)^{A}	0.251 ± 0.002 (19)^{A}
P0206x2>GFPx2	0.108 ± 0.001 (24)^{A}	0.170 ± 0.002 (14)^{A}	0.259 ± 0.002 (15)^{AB}
P0206>Dp110	0.107 ± 0.001 (10)^{A}	0.161 ± 0.001 (44)^{B}	0.253 ± 0.003 (15)^{A}
P0206>PTEN	0.108 ± 0.001 (30)^{A}	0.190 ± 0.002 (23)^{C}	0.271 ± 0.007 (12)^{B}
phm: PG			
phm>GFP	0.105 ± 0.001 (20)^{A}	0.169 ± 0.001 (28)^{A}	0.259 ± 0.002 (20)^{A}
phm>GFPx2	0.104 ± 0.001 (15)^{A}	0.171 ± 0.001 (13)^{A}	0.255 ± 0.003 (34)^{A}
phm>Dp110	0.106 ± 0.001 (30)^{A}	0.160 ± 0.001 (45)^{B}	0.241 ± 0.002 (43)^{B}

The first row for each GAL4 line indicates in which tissues GAL4 drives its expression. PG is the prothoracic gland, and CA is the corpora allata. Means indicated by different letters (uppercase superscript) were found to be statistically significant at $p < 0.05$ by ANOVA and a post-hoc comparison of the means with a Tukey HSD. The values after the means are the standard errors of the means. The number of individuals measured is in parentheses.

starved earlier than 11.5 hr AL3E is unclear. The morphology of phm>Dp110 wandering L3 imaginal discs appeared normal, and they were competent to undergo some of the earliest metamorphic processes such as eversion and elongation. Furthermore, *wingless* and *senseless* protein expression pattern in the wing discs appeared grossly normal, indicating that patterning of the wing pouch and sensory cells, respectively, was occurring more or less normally in animals starved before 11.5 hr AL3E (data not shown). Animals starved at 4 hr AL3E exhibited molting defects in the prepupal stages, but individuals starved at 8 hr AL3E did not (data not shown).

Interestingly, starvation after critical weight has been reached accelerated the time to metamorphosis. For control phm>GFP larvae, starved larvae pupariated more rapidly than larvae that were allowed to continue feeding (Figure 6D). Similarly, phm>GFPx2 larvae pupariated 10 hr earlier when starved. Fed phm>GFPx2 larvae pupariated 1.85 hr later than fed phm>GFP larvae (data not shown). Starvation also accelerated the time to pupariation in phm>Dp110 larvae, but only in the smallest larvae. Phm>Dp110 larvae starved after 19 hr AL3E and weighing more than 0.7 mg took the same time to pupariate as fed larvae.

Ecdysteroid Titers for Larvae with Enhanced or Suppressed PG Growth

To explore whether phm>Dp110 larvae reached critical weight earlier as a result of enhanced ecdysteroidogenesis, we examined ecdysteroid titers in early feeding L3 larvae around the time of attainment of critical weight. We could not detect a significant difference in ecdysteroid titers between phm>Dp110 larvae and controls at either 4 or 13 hr AL3E (Figure 7A). Larvae in which PG growth was suppressed (P0206>PTEN) also showed no difference from controls in ecdysteroid titer either at 0 hr (data not shown) or 22 hr AL3E (Figure 7B) but had reduced ecdysteroid concentrations in comparison to controls at 44 hr AL3E.

Discussion

By manipulating the growth of the PG, we have been able to investigate the role of this gland during three size-assessment milestones: threshold size for metamorphosis, minimal viable weight, and critical weight.

PG, Larval Growth Rate, and Threshold Size for Metamorphosis

Our manipulations of insulin-dependent PG growth showed that this growth is inversely related to larval growth. Suppressing the growth of the PG (P0206>PTEN) produced larvae that spent more time in each instar and were larger than normal. These effects are presumably due to a combination of reduced ecdysteroid biosynthesis, which is known to delay development [31–33], and increased growth rate, as was shown by Colombani et al. [34]. Conversely, larvae with enlarged PGs (phm>Dp110) showed accelerated development in the L3. Their growth rate was dependent on nutritional conditions. Whereas phm>Dp110 larvae reared on suboptimal food grew slowly, well-fed phm>Dp110 larvae grew at the same rate as controls. Together, these data indicate that the growth of the PG negatively regulates the growth rate of the whole animal and that this regulation is modulated by nutrition.

In addition, decreasing PG size in P0206>PTEN larvae resulted in premature metamorphosis and the formation of L2 puparia. Similar L2 puparia have been described in larvae with mutations that affect the regulation of ecdysteroid biosynthesis or signaling [31–33] and in larvae where the Broad isoform Z3 was overexpressed in the ring gland, resulting in its apoptosis [3]. L2 puparia are seen in situations where ecdysone synthesis is compromised because larvae cross the threshold weight for metamorphosis prior to the production of sufficient ecdysone to initiate a larval molt, redirecting their development to the metamorphic pathway [3].

Reducing PG size resulted in reduced ecdysteroid biosynthesis; P0206>PTEN larvae showed reduced ecdysteroid titers at 44 hr AEL3, and phm>PTEN larvae only molted to L2 when fed 20E. Under conditions of low ecdysteroid synthesis, fast-growing larvae could surpass the threshold for metamorphosis before the ecdysteroid titer was sufficient to induce a molt, resulting in L2 prepupae. Slower-growing larvae would be unable to reach this threshold weight before the rise in ecdysteroid titer induced the molt to L3. Indeed, under-

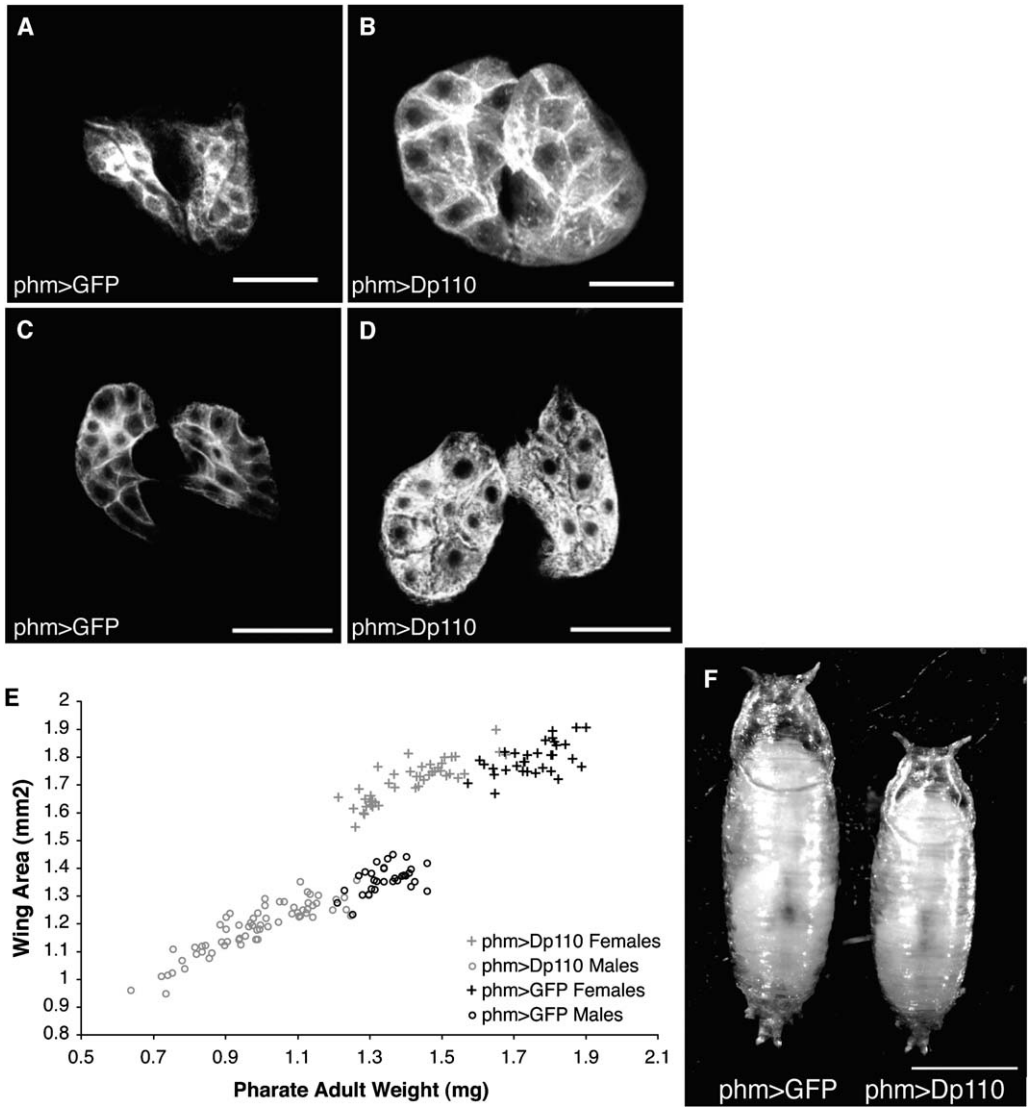


Figure 3. Enhancing Prothoracic-Gland Growth Decreases Adult Size

(A and B) GFP expression in the prothoracic glands (PGs) of control (phm>GFP) first instar (L1) larvae and L1 larvae with enlarged PG (phm>Dp110), respectively.

(C and D) GFP expression in the PGs of early third instar (L3) larvae from control cultures (phm>GFP, [C]) and from larvae with enlarged PGs (phm>Dp110, [D]).

(E) Adult wing area as a function of pupal weight for phm>GFP and phm>Dp110 males and females.

(F) Puparia formed from phm>GFP (left) and phm>Dp110 larvae (right). The scale bars represent 20 μm in (A) and (B), 50 μm in (C) and (D), and 1 mm in (F).

nourished, and presumably slow-growing, P0206>PTEN L2 larvae all molted to L3, whereas only 33% of the well-fed P0206>PTEN larvae molted to L3.

PGs and Minimal Viable Weight

Enlarging the PG of larvae reared under constant light caused larvae to initiate metamorphosis earlier and at smaller sizes. Nevertheless, even though larvae starved early after the L3 molt were able to pupariate, they were unable to survive to pupation unless they had fed for at least 11.5 hr. This suggests that phm>Dp110 larvae starved prior to 11.5 hr AL3E initiated metamorphosis before surpassing the minimal viable weight. Furthermore, although in control larvae, critical weight and minimal viable weight are apparently attained at the same time, they are uncoupled in phm>Dp110 larvae. Therefore, the assessment of critical weight is dependent on PG growth, whereas the minimal viable weight is not.

PGs and Critical Weight

In *Drosophila*, the PGs are responsible for a size-assessment event, early in the L3, that induces the onset of metamorphosis once critical weight is surpassed. Enhancing PG growth resulted in an overestimation of body size, thereby causing the larva to initiate metamorphosis early, at a subnormal size. Under LL, the effects of enlarging the PG were enhanced, producing

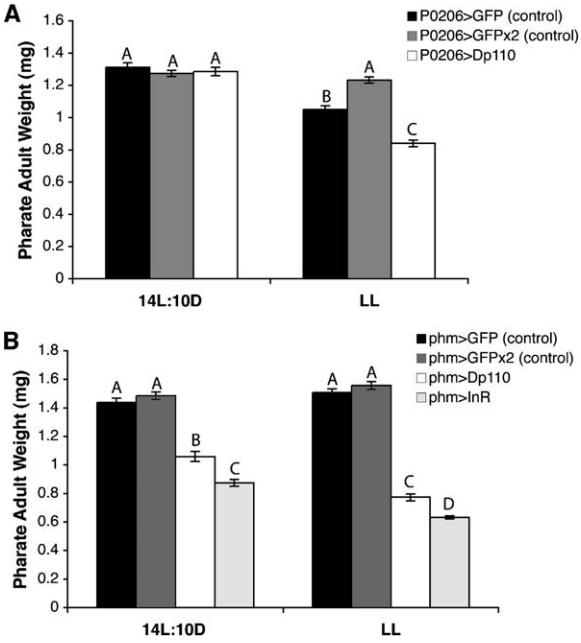


Figure 4. Photoperiod Affects Pharate-Adult Weight in Individuals with Enlarged Ring Glands

(A) The effect of light regime (14 hr light, 10 hr dark [14L: 10D] or constant light [LL]) on pharate-adult weight in P0206>GFP, P0206>GFPx2, and P0206>Dp110 larvae (n = 40–84).

(B) The effect of light regime on pharate adult weight in phm>GFP, phm>GFPx2, phm>Dp110, and phm>InR individuals (n = 30–76). For both (A) and (B), the treatments not sharing the same letter are significantly different ($p < 0.007$), as determined by ANOVA and a post-hoc Tukey HSD comparison of the means. The error bars represent standard errors of the means.

individuals that pupariated even earlier at even smaller sizes, suggesting that when PTTH release was unconstrained by circadian gating [8], the PTTH delay period was reduced. These data provide the first indication in *Drosophila* that the post-critical-weight PTTH release may be under photoperiod control, as it is in *Manduca* [8].

There has been some discussion in the literature as to whether critical weight as described in *Drosophila* is the same as critical weight as defined in *Manduca* [9]. This discussion has arisen because the definition for *Manduca* states that critical weight is the minimal size at which starvation can no longer delay the onset of metamorphosis [4], but when *Drosophila* larvae are starved before critical weight is reached, they die [5, 6, 35]. Our data suggest that this is due to a tight relationship between minimal viable weight and critical weight in *Drosophila*. Effects more similar to those observed in *Manduca* can be obtained when pre-critical-weight *Drosophila* larvae are starved for an interval and then refed. Under these conditions, they delay metamorphosis for a period greater than the period of starvation [5]. Thus, critical weight in *Drosophila* appears to agree with the definition provided by Nijhout and Williams [4]. Much of the confusion surrounding critical weight in *Drosophila* has arisen because in wild-type larvae, minimal viable weight and critical weight are achieved at the same time.

After critical weight has been surpassed, the metamorphic pathway appears to be partially suppressed by continued feeding in *Drosophila* ([5, 36] and this study). Hence, the nutrition pathway appears to promote growth and suppress metamorphosis, whereas insulin-dependent PG growth suppresses larval growth and promotes differentiation.

PG Growth and the Mechanism for Size Assessment

The effects of increased growth in the PG are not simply due to increasing cell size, but rather are specific to the nutrition-dependent InR signaling pathway. Studies conducted by Colombani and coauthors [34] indicate that when either dMYC or cyclinD/cdk4 are used to enlarge the PG cells, there is no reduction in overall body size. Overexpression of dMYC, of cyclinD/cdk4, and of Dp110 all enhance cell growth, but they do so in fundamentally different manners by using separate cascades [37]. Whether the size-assessment mechanism oper-

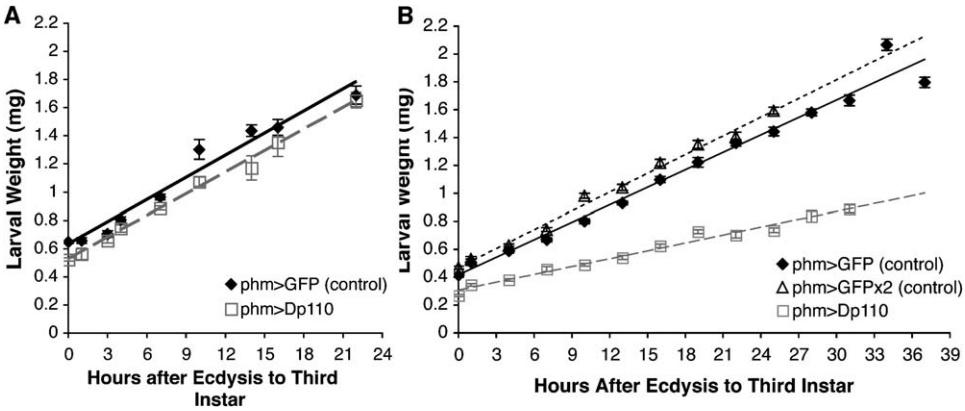


Figure 5. Growth of Third Instar (L3) Larvae with Enlarged Prothoracic Glands under Constant Light with and ithout Yeast Supplement

(A) Growth during the first 22 hr of the L3 for larvae with enlarged prothoracic glands (PGs) (phm>Dp110) as compared to controls (phm>GFP) when supplemented with yeast paste (n = 7–45 larvae).

(B) Growth rates of L3 larvae with enlarged PGs (phm>Dp110) as compared to those of control phm>GFP and phm>GFPx2 larvae when reared without yeast supplement (n = 24–46 larvae). The error bars represent standard errors of the means.

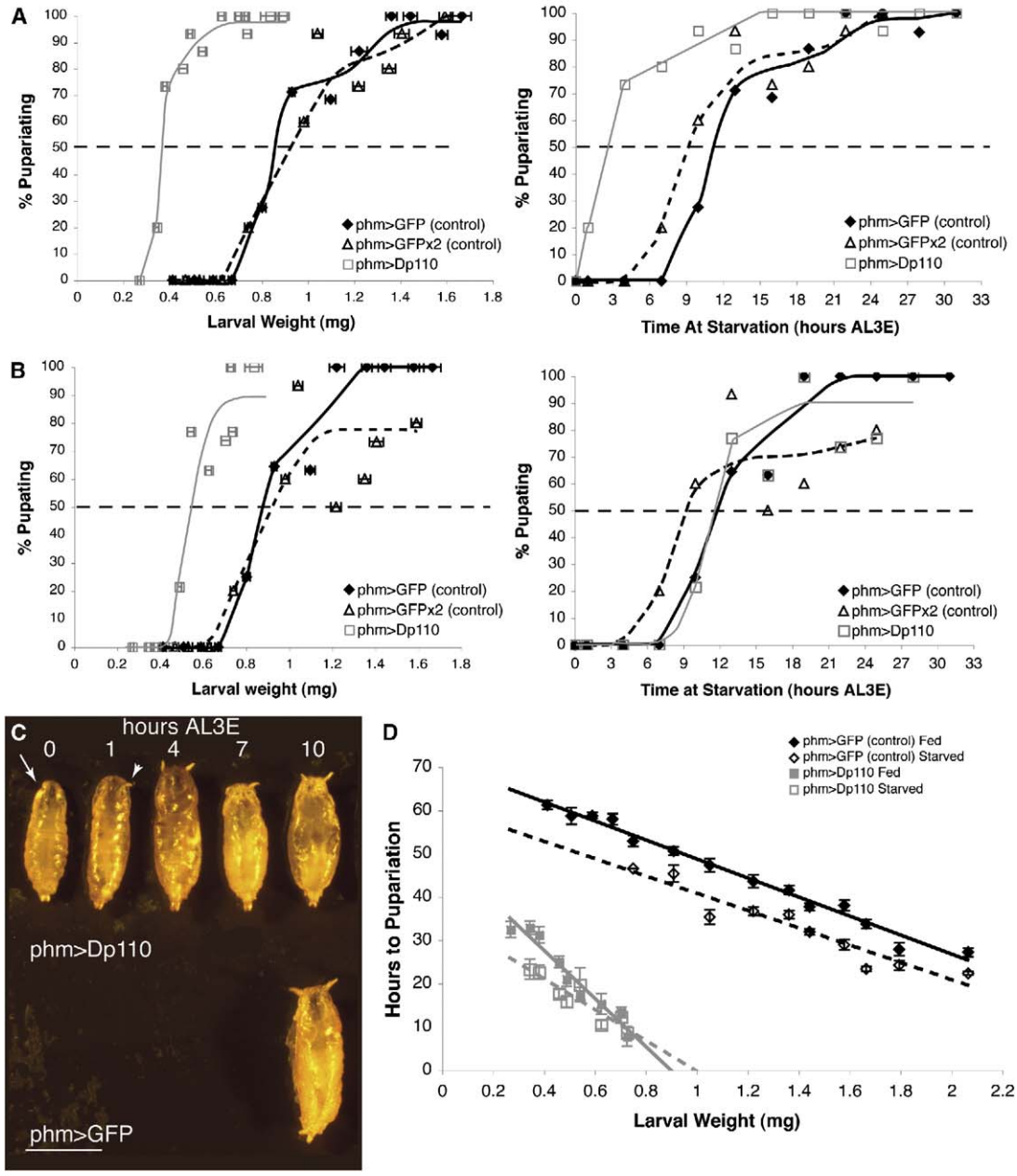


Figure 6. Larvae with Enlarged Prothoracic Glands Pupariated Earlier and Showed Reduced Critical Weight but Surpassed Minimal Viable Weight at the Same Time as Controls

(A) Percent of larvae that underwent pupariation after starvation at a given size and time after ecdysis to third instar (L3) for phm>GFP (closed symbols, black), phm>GFPx2 (open symbols, black), and phm>Dp110 (open symbols, gray) larvae (n = 9–45 individuals per data point). The horizontal dashed line is the threshold where 50% of the larvae pupariated.

(B) Percent larvae that underwent pupation after starvation at a given size and time after ecdysis to L3. The horizontal dashed line is the threshold where 50% of the larvae survive to pupation.

(C) Larval/prepupal intermediates or puparia formed from either phm>Dp110 larvae or control phm>GFP larvae starved at various times after L3 ecdysis (AL3E). A phm>Dp110 larva starved 0 hr AL3E formed a larval/prepupal intermediate that tanned but did not contract properly (arrow). A tiny puparium formed by a phm>Dp110 larva starved at 1 hr AL3E shows the everted anterior spiracles (arrowhead) and has also formed an operculum. Phm>GFP larvae did not pupariate under starvation conditions unless they had been fed for at least 10 hr AL3E. The scale bar represents 1 mm.

(D) Time to pupariation as a function of size for both starved and fed phm>Dp110 and phm>GFP individuals. Data points represent the mean of 7 to 15 individuals for the fed treatments and the mean of 1 to 15 individuals for the starved treatments because the smallest size classes rendered very few pupae when starved. Error bars represent standard errors of the means.

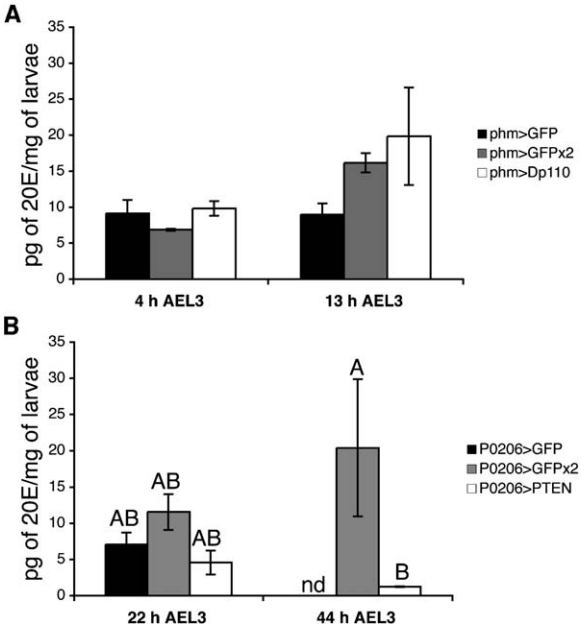


Figure 7. Ecdysteroid Titers in Larvae with Enhanced or Suppressed PG Growth

(A) Ecdysteroid titers measured for phm>GFP, phm>GFPx2, and phm>Dp110 L3 larvae at 4 and 13 hr after third instar (L3) ecdysis (AL3E); (B) Titers of P0206>GFP, P0206>GFPx2, and P0206>PTEN L3 larvae at 22 hr AL3E and of P0206>GFPx2 and P0206>PTEN L3 larvae at 44 hr AL3E (nd = not determined). Values are expressed as the means of 20E equivalents from three to six samples of ten larvae each, except for phm>GFPx2 at 4 hr AL3E, where only two samples were obtained. Error bars represent standard errors of the means. For (B), the treatments not sharing the same letter are significantly different ($p < 0.05$), as determined by ANOVA and a post-hoc Tukey HSD comparison of the means.

ates via increased intracellular PIP_3 levels in the PG cells or the accumulation of some other downstream component of the InR cascade in these cells is unknown.

Although we were unable to detect any difference in ecdysteroid titers in larvae with enlarged PGs, there is evidence that increased InR signaling in the PG cells can produce mild increases in ecdysteroidogenesis and ecdysone signaling, increases that are below the level of detection of ecdysteroid-titer assays. Larvae with enlarged PGs showed both a mild increase in the transcription of *phantom* during feeding stages [34] and an increase in the transcription of the early ecdysone response gene E74B ([34]; see also [38] in this issue of *Current Biology*. These subtle differences in ecdysteroid titers may be important for determining growth rates and for size assessment. A gradual rise in ecdysteroid titers is coincident with the time that critical weight is reached in *Drosophila* [39, 40]. Also, in *Precis*, subtle shifts in 20E concentrations are important for growth. Basal concentrations of 20E in combination with bombyxin enhance the growth of wing imaginal tissues in vitro; slightly higher concentrations of 20E suppress growth [23].

Mutations that cause imaginal disc and larval overgrowth often cause delayed pupariation and, in some cases, show low L3 ecdysteroid titers [41]. In the case of the mutant *lethal (2) giant larvae*, the ring glands are smaller than normal and have the ultrastructural appearance of glands that have low rates of ecdysteroid biosynthesis [42]. Delayed pupariation in these larvae can be rescued by implanting wild-type ring glands [43]. Lastly, hypomorphic mutations in *DHR4*, a repressor of ecdysone-induced early genes, cause reductions in critical weight and early-pupariation phenotypes similar to what we have described [44]. Thus, the size-assessment mechanism is likely to involve surpassing a threshold ecdysteroid titer above which the activation of the ecdysone cascade occurs.

These data allow us to construct the following model for size assessment in *Drosophila*. As PG cells grow in response to increased InR signaling, they increase their basal level of ecdysteroid biosynthesis. Critical weight is then reached when systemic ecdysteroid concentrations surpass a threshold that sets into motion the endocrine events that will end the growth phase of larval development and allow the larva to begin metamorphosis.

Conclusions

Studies in the mid-1970s defined a size-assessment event during the final instar of the moth *Manduca sexta*; termed critical weight, it is the minimal size required for the timely initiation of metamorphosis. How insect larvae determine when they have reached critical weight has long been a mystery. We hypothesized that a size-assessing tissue determines when critical weight had been reached. Suppressing growth in this size-assessing tissue would cause an underestimation of body size, resulting in metamorphosis at larger than normal sizes, whereas enlarging this tissue would result in subnormal sizes. Our studies in *Drosophila* have shown that manipulation of the growth of the PG via the InR pathway produced these types of effects. Furthermore, larvae with enlarged PGs metamorphosed at even smaller sizes when reared under LL, suggesting a role for PTTH circadian gating in this response. Smaller size arose both as a result of a reduction in growth rate, an effect that could be rescued via nutritional supplementation, and the early onset of metamorphosis. Most importantly, larvae with enlarged PGs had a remarkably reduced critical weight, suggesting that they are severely overestimating their own body size. These results offer a very new perspective on the problem of size control in insects, uniting the recent data exploring the role of nutrition and the insulin-receptor pathway on growth with the classical physiological experiments that defined critical weight.

Experimental Procedures

Fly Stocks

Three GAL4 lines were used to manipulate PG and CA size: *phantom GAL4,UAS mCD::GFP/TM6B Tb* (gift from Michael O'Connor), *Aug21/CyO GFP* ([45]; gift from G. Korge), and *P0206,UAS mCD::GFP* [26]. These lines were crossed to one of six stocks: *yw* (generating >GFP controls for phm and P0206), *yw;P0206,UAS mCD8::GFP* (for the P0206x2>GFPx2 controls), *yw;UAS mCD8::GFP* (generating the >GFPx2 controls for phm and P0206 and the >GFP controls for Aug21), *yw;UAS PTEN (III),hs flp;UAS Dp110*, or *yw flp;UAS-InR$^{29.4}$ (III)* (the latter three are gifts from Bruce Edgar).

Insect Cultures

Flies oviposited on yeasted apple-juice plates. Eggs were then distributed onto 60 × 15 mm petri dishes filled with approximately 10 ml cornmeal/molasses medium, with 200 eggs per plate. For every cross, three to six replicates were plated. For the initial size descriptions, larvae received yeast-paste supplement. All other experiments were conducted without this supplement unless otherwise noted. Larvae raised on food containing 50% of the normal nutrients were fed a mixture of 50% cornmeal/molasses medium and 50% agar solution (2% agar in water). Unless otherwise stated, larvae were reared at 25°C under 14L:10D cycle and were observed three times daily, in the case of phm larvae, or once daily, in the case of P0206 larvae, to assess the duration of each instar. When larvae reached the third instar, the food plates were taped into a 100 mm dish to ensure sufficient room for pupariation.

Larvae to be fed 20E were counted onto food plates as described above. Two days after hatching, 20 first-instar larvae were transferred onto a small food plate (35 × 10 mm) containing cornmeal/molasses medium, and the remainder were transferred onto a small food plate containing 1 μg 20E (Sigma)/ml of food (stock solution: 1 mg/ml 20E in isopropanol).

Measurements of Growth

For mouth-hook measurements, a sample of five to ten larvae from each instar was removed from each food plate. The mouth hooks were mounted in 100% glycerol.

Pharate adults were collected from food plates, cleaned off by immersion in 100% bleach for 5 min, and individually weighed on a Mettler M5 microbalance. Each pharate adult was transferred into a 5 ml plastic or glass test tube, containing a plug of moistened cotton at the bottom of the tube and stoppered with another piece of cotton.

Emerged adults were preserved in 95% ethanol, and their wings were mounted in 100% glycerol for measurement. Mouth-hook and wing images were captured with a Photometrics Coolsnap cf camera mounted on a Nikon Eclipse E600 compound microscope and measured in ImageJ. Wing areas were measured starting from the intersection between the second and third wing vein and measuring around the perimeter of the wing to end at the same point.

Determination of Critical Weight

Larvae were cultured as mentioned above, but under a constant-light regime. Larvae that ecdysed from L2 to L3 over a 2 hr period were placed onto new food plates and weighed at 3 hr intervals on a Mettler M5 microbalance. Each weighed sample was divided into fed and starved treatment groups. Fed animals were placed on small food plates. Starved larvae were transferred to same-sized dishes filled with a medium of 2% agar in water. Larvae were observed every 4 hr during the day, with an 8 hr interval at night.

Propidium-Iodide Staining of Ring Glands

Larvae were dissected and fixed in 4% formaldehyde in phosphate buffered saline (PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0) for 20 min at room temperature. After fixation, the tissue was washed in PBT (PBS and 1% Triton X) and incubated in a solution containing propidium iodide (1 ng/ml), DNase-free RNase (5 ng/ml, to eliminate cytoplasmic background due to RNA), and 2% normal donkey serum in PBT overnight at 4°C. After they were rinsed with PBT, ring glands were mounted on poly-L-lysine-coated coverslips in Fluoromount-G (Southern Biotech) and imaged with either a Biorad MRC-600 Confocal Microscope or a Biorad Radiance Confocal Microscope.

Enzyme Immunoassay for Ecdysteroid Titers

Ecdysteroid titers were measured according to the protocol in Porcheron et al. [46]. In brief, animals were synchronized by collecting newly ecdysed L3 larvae every 2 hr. A sample of ten larvae was weighed on a Mettler M5 microbalance and then preserved in methanol. Prior to assaying, the samples were homogenized and centrifuged, and the resulting methanol supernatant was dried. Samples were resuspended in 50 μl of enzyme immunoassay (EIA) buffer (0.4 M NaCl, 1 mM EDTA, 0.1% bovine serum albumin, and 0.01% sodium azide in 0.1M phosphate buffer). 20E EIA antiserum and 20E acetylcholinesterase tracer were purchased from Cayman Chemicals.

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